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High levels of Cellular Prion Protein improve astrocyte development

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ABSTRACT

Prion protein (PrP^C) has neuroprotective functions and herein we demonstrate that astrocytes from PrP^C-over-expressing mice are more resistant to induced cell death than wild-type astrocytes. The Stress-Inducible-Protein 1 (STI1), a PrP^C ligand, prevents cell death in both wild-type and PrP^C-over-expressing astrocytes through the activation of protein-kinase-A. Cultured embryonic astrocytes and brain extracts from PrP^C-over-expressing mice show higher glial fibrillary acidic protein expression and reduced vimentin and nestin levels when compared to wild-type astrocytes, suggesting faster astrocyte maturation in the former mice. Our data indicate that PrP^C levels modulate astrocyte development, and that PrP^C–STI1 interaction contributes to protect against astrocyte death.

1. Introduction

Prions are responsible for transmissible spongiform encephalopathies (TSEs). The mechanism of disease propagation involves the interaction of prion scrapie (PrP^{Sc}) with its cellular isoform, prion protein (PrP^C), and the subsequent abnormal structural conversion of the latter [1,2]. The massive neurodegeneration present in these diseases has been associated with the gain of neurotoxic activity of PrP^{Sc} [3,4]. On the other hand, several cellular functions have been assigned to PrP^C suggesting that its loss-of-function may be an important component for the pathogenesis of prion diseases [3,5].

PrP^C is highly expressed in the brain and plays several functions during brain development [1,6–8]. A great number of investigations concentrated in uncovering the neuronal functions of PrP^C, while its role in glial cells has been poorly addressed. PrP^C is found in cell surface, within cytoplasm, and it can also be secreted [9–11]. This protein has been related to a wide range of neuronal functions [9,11–20]. PrP^C regulates neuronal polarization by β1 integrin activity and shows interaction with fibronectin and cytoskeleton dynamics [21]. In addition, PrP^C acts as a receptor for the laminin γ-1 chain and the transmembrane protein neuronal cell adhesion molecule (NCAM), which mediates neuronal adhesion and neurite outgrowth [19,20,22]. In vivo studies showed that interactions of PrP^C with laminin γ-1 chain or the Stress Inducible Protein 1 (STI1), another PrP^C ligand, are involved with memory consolidation [23,24].

In neuron-glia interaction context, it has been shown that glutamate uptake from astrocytes is dependent on PrP^C expression, which may influence neuronal survival [25]. In addition, PrP^C binds an astroglial Na1/K1-ATPase involved in lactate transport by astrocytes [26]. In neuron-astrocyte co-cultures, astrocytes from wild-type (WT) mice promoted a higher level of neuritogenesis than astrocytes obtained from PrP^C-null animals. Laminin secreted and deposited at the extracellular matrix by WT astrocytes shows a fibrillar organization and promotes neurite outgrowth, while laminin secreted from PrP^C-null astrocytes has a punctate pattern and it is less permissive to neuronal differentiation [11]. Moreover, both PrP^C and STI1 are secreted by astrocytes and support neuritogenesis and neuronal survival [9,11]. Indeed, PrP^C expression in astrocytes is critical for sustaining essential mechanisms required for neuronal differentiation and survival.

Recently, it has been demonstrated that PrP^C protects an astrocyte cell line derived from PrP^C-null mice from oxidative stress [27]. We also verified that STI1 prevents cell death and induces morphological changes in WT astrocytes, whereas PrP^C-null astrocytes are not affected by STI1 treatment, indicating a further role of PrP^C also in glial development [5].

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In the present study, we demonstrate that the PrP^C over-expression in the brain increases astrocyte resistance to cell death. STI1 prevented cell death in both WT and PrP^C over-expressing (Tg20) astrocytes through the protein kinase A (PKA) pathway. We also demonstrate that astrocytes show increased glial fibrillary acidic protein (GFAP) expression and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinases 1 and 2 (ERK1/2) activity, as well as reduced vimentin and nestin expression, suggesting a faster rate of astrocyte maturation in PrP^C over-expressing mice.

2. Materials and methods

The experimental approach used in the manuscript is detailed in Fig. 1.

2.1. Animals

Our experiments were performed with a line of *PrP^C over-expressing* mice (*Tg20*) originally described by Fischer et al. [28]. *Tg20* mice carry a half genomic construct of the mouse *a* allele which expresses 5–8 times more PrP^C than the WT mice [28]. The transgene was introduced into mice deficient in PrP^C expression ($Prnp^{0/0}$) that were generated by Bueler et al. [29]. The WT mice used ($Prnp^{+/+}$) in the present study were descendants of a F1 generation mouse produced by interbreeding the original parental strains (C57/BL/6J and 129/Sv mice) used to generate $Prnp^{0/0}$ mice. The WT mice express the allele *a* [30]. *Tg20* mice were provided by Dr. Charles Weissmann (Scripps Florida, Jupiter, FL, USA). The Code of Ethics of the EU Directive 2010/63/EU for animal experiments was strictly followed for all experiments. This study was approved by the local Animal Care and Use Committee at the A.C. Camargo Hospital.

2.2. Astrocyte culture

Primary astrocyte cultures were prepared as previously described [11] from the cerebral hemispheres of embryonic day 18 (E18) WT ($Prnp^{+/+}$) and *PrP^C over-expressing* mice (*Tg20*). 3×10^5 astrocytes (10 cm-plate with 10 ml) were grown in Dulbecco's Modified Eagle Medium (DMEM) enriched with 10% fetal calf serum (FCS, Invitrogen-Life Technologies – NY) until confluence.

2.3. Cell death assay

Mouse recombinant STI1 (His6–STI1) and STI1$\Delta_{230-245}$ (His6–STI1$\Delta_{230-245}$) were purified as described previously [5,9,31]. Recombinant STI1 was quantified for bacterial lipopolisaccharide (LPS) contamination by the Limulus Amebocyte Lysate test (Cambrex), and samples were purified by detoxi-gel endotoxin removing gel (DGRG; Thermo Scientific Pierce) before use [5]. After confluence, primary astrocyte cultures were pre-incubated in serum free DMEM with STI1 (0.05, 0.5, 1.0 or 2.0 μM), STI1$\Delta_{230-245}$ (1.0 μM) or control buffer consisting of tris-buffered saline (TBS) for 2 h, followed by staurosporine (25, 50, or 100 nM) for 16 h. The signaling pathway inhibitors ERK1/2 inhibitor (UO126), PKA inhibitor (KT5720), protein kinase C (PKC) inhibitor (chelerythrine chloride, chel) and phosphatidylinositol 3-kinase (PI3K) inhibitor (LY294002) (Calbiochem) were added 1 h before incubation with STI1. The cell cultures were fixed with 4% paraformaldehyde and stained with propidium iodide (0.5 μg ml^{-1}; Sigma). Cell death induced by staurosporine was detected as condensed and pyknotic profiles and the number of pyknotic nuclei was quantified in 10 microscopic fields [5].

2.4. Immunoblotting analysis

Cells were cultured until confluence, the culture medium was changed to DMEM with 1% FCS, and STI1 (0.5 μM) was added every day for 5 days. Cells were incubated for an additional 5 days and lysed as previously described [5]. Western blotting assays were conducted using rabbit anti-GFAP (1:1000), mouse anti-vimentin (1:1000), mouse anti-nestin (1:1000) (Chemicon Int.) and rabbit anti-β-actin (1:200; Sigma) antibodies. Rabbit or mouse pre-immune serums were used as negative controls. The bands obtained after X-ray film exposure to the membranes were analyzed by

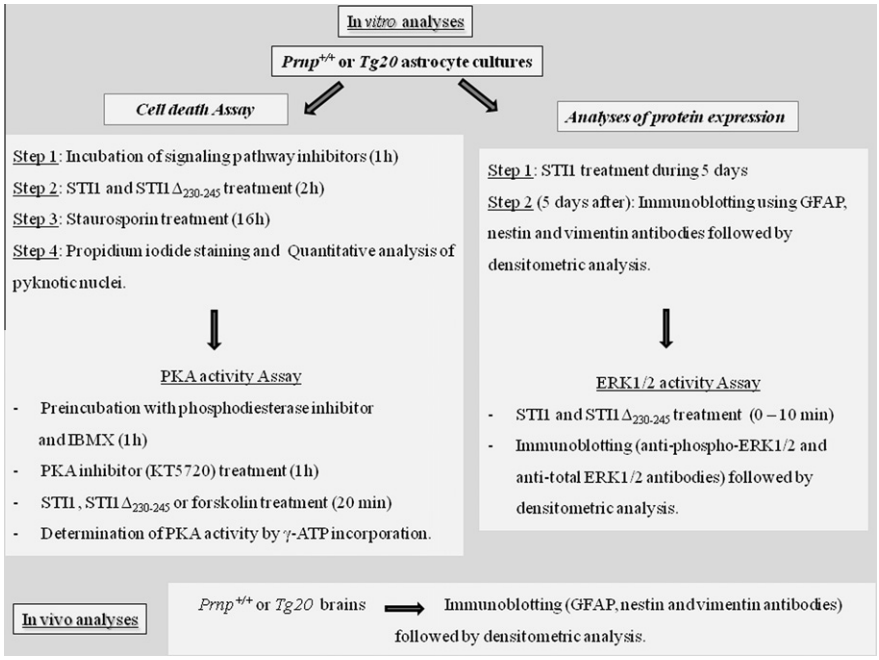

Fig. 1. Summary of experimental approach. $Prnp^{+/+}$ and Tg20 astrocytes were submitted to cell death and PKA activity assays. Immunoblotting analysis using GFAP, nestin, vimentin, phospho-ERK1/2 and total ERK1/2 antibodies were performed in brain extracts and cultured astrocytes from $Prnp^{+/+}$ and Tg20 mice. The STI1 effect was investigated under these conditions.

densitometric scanning and quantified using Scion Image software. Values represent the ratio between GFAP, vimentin or nestin and actin for each sample. Values from untreated *Prnp*$^{+/+}$ astrocytes were set as 1.0, and the others are relative to it. E18 brains were lysed in the same buffer as the astrocytes, and SDS–PAGE and immunoblotting were performed using the same conditions described above.

2.5. Kinase assays

2.5.1. PKA activity

For determination of PKA activity, confluent astrocytes were preincubated with a phosphodiesterase inhibitor (Calbiochem), 3-isobutyl-1methylxantine (IBMX; Calbiochem) (100 μM) for 1 h at 37 °C and treated with or without PKA inhibitor KT5720 (0.06 μM) for 1 h at 37 °C. STI1 and STI1$\Delta_{230–245}$ (1.0 μM) or forskolin (10 μM) (LC Laboratories) were added to the cultures for 20 min at 37 °C. The cells were then lysed and the PKA activity was determined by y-ATP incorporation into a PKA-specific substrate provided by the appropriate protein kinase assay system kit (Upstate Biotechnologies). The reactions were performed according to the manufacturer's instructions.

2.5.2. ERK1/2 phosphorylation

Phosphorylation assays were conducted using the PhosphoPlus p44–42 MAPK (Thr202/Tyr204) antibody kit (Cell Signaling) according to the manufacturer's instructions. After confluence, astrocytes were stimulated with STI1 or STI1$\Delta_{230–245}$ (0.5 μM) for different incubation periods and lysed in Laemmli buffer. Cell extracts were subjected to SDS–PAGE, followed by immunoblotting with anti-phospho-ERK1/2, and anti-total ERK1/2 antibodies. The bands obtained after X-ray film exposure to the membranes were analyzed by densitometric scanning and quantified using Scion Image software.

2.5.3. Statistical analyses

The results represent the mean ± S.D. of at least four independent experiments conducted in triplicate. Statistical analyses were performed by ANOVA followed by the Tukey post hoc test. The phospho-ERK1/2 statistical analyses were conducted using a single-mean Student's *t* test.

3. Results

3.1. PrP^C expression modulates astrocytes survival through interaction with STI1 via PKA

Prnp$^{+/+}$ *(WT)* and *Tg20 primary astrocyte cultures* show similar growth in vitro. However, they are more resistant to cell death induced by staurosporine (Fig. 2A), indicating that *PrP*^{C} over-expression protects astrocytes from cell death.

The PrP^C-ligand STI1 prevented both *Prnp*$^{+/+}$ and Tg20 astrocytes from staurosporine-induced death in a dose-dependent manner (Fig. 2B). Additionally, STI1$\Delta_{230–245}$, which lacks the PrP^C binding site, did not promote survival in astrocytes, confirming that the protection mediated by STI1 depends on its specific interaction with PrP^C (Fig. 2C). The protective effect of STI1 was abrogated by a PKA inhibitor (KT5720), whereas PKC (Chel), PI3K (LY294002) and ERK1/2 (U0126) inhibitors had no effect (Fig. 2C). STI1 treatment induced similar PKA activation in both *Prnp*$^{+/+}$ and Tg20 astrocytes, indicating that PKA saturation is achieved even with lower PrP^C levels. Similar PKA activation levels were also observed when either *Prnp*$^{+/+}$ or *Tg20* astrocytes were treated with forskolin, an adenylate cyclase activator. The addition of STI1$\Delta_{230–245}$ or STI1 plus a PKA inhibitor had no effect on PKA activity in both types of astrocytes (Fig. 2D). These results indicate that PrP^C levels are directly correlated with resistance to cell death and suggest the involvement of the PKA pathway in the STI1/PrP^C-dependent protection against astrocyte death.

3.2. PrP^C levels modulate GFAP, vimentin and nestin expression during brain development

Astrocyte differentiation was evaluated by assessing GFAP, vimentin and nestin expression. GFAP and vimentin are intermediate filament proteins whose expression is regulated during astrocyte development [32–35]. The expression of nestin, another intermediate filament protein and stem cell marker, is downregulated during astrocyte maturation [36]. Interestingly, under basal conditions, *Tg20* astrocytes expressed more GFAP (Fig. 3A) and less vimentin (Fig. 3B) and nestin (Fig. 3C) than WT cells, indicating that Tg20 cells were more differentiated at this embryonic stage than their WT counterpart.

When WT astrocytes were treated with STI1, GFAP expression increased (Fig. 3A), while vimentin (Fig. 3B) and nestin (Fig. 3C) levels decreased. Conversely, no effect on the basal levels of these proteins was observed when *Tg20* astrocytes were treated with STI1 (Fig. 3A–C). These results confirm the role of STI1 in astrocyte differentiation and indicate that in cells that overexpress PrP^C, differentiation was prematurely achieved.

Similarly to primary cultures, brain extracts from the cerebral cortex, cerebellum and hippocampus of E18 Tg20 embryos showed higher GFAP expression (Fig. 4A), and lower vimentin (Fig. 4B) and nestin levels (Fig. 4C) when compared to WT E18 mice. In particular, GFAP expression was almost threefold higher in *Tg20* mouse embryo cerebellums relative to *Prnp*$^{+/+}$, confirming that PrP^C is involved in astrocyte differentiation in the developing brain.

3.3. ERK1/2 activity is increased in PrP^C over-expressing astrocytes

STI1 is able to induce ERK1/2 activation in astrocytes (Fig. 5A), while no ERK1/2 activation was observed in *Prnp*$^{+/+}$ astrocytes treated with STI1$\Delta_{230–245}$ (Fig. 5B), which confirms our previous data [5] that ERK1/2 activation is mediated by PrP^C–STI1 interaction. Interestingly, *Tg20* astrocytes presented at least fourfold higher ERK1/2 basal activity, when compared to WT cells (Fig. 5B). Conversely, STI1 is unable to activate ERK1/2 in *Tg20 astrocytes* indicating that the activation of this enzyme should reach a plateau within these cells.

4. Discussion

The data presented here provide evidence that PrP^C over-expression promotes resistance to cell death, as well as early astrocyte maturation. In this study, we demonstrated that PrP^C over-expression protects astrocytes from cell death induced by staurosporine, an alkaloid isolated from the bacterium *Streptomyces staurosporeus*, which is known to induce apoptosis in many cell lines through caspase-3 activation [37–40].

In fact, Tg20 mice are less susceptible to age-related alterations in locomotor, anxiety-like responses and short-term social recognition memory [41]. Moreover, the upregulation of PrP^C expression after cerebral ischemia and hypoxia exerts a neuroprotective effect on injured neural tissue [42]. On the other hand, our group has demostrated that PrP^C-null astrocytes were more sensitive to staurosporine-induced cell death than WT cells [5]. Recently, it was demonstrated that the ectopic expression of PrP^C protects PrP^C-null astrocyte cell lines from oxidative stress [27]. In addition, it is known that PrP^C-null neurons are more susceptible to stress conditions, such as serum deprivation [13], Bax expression [14]

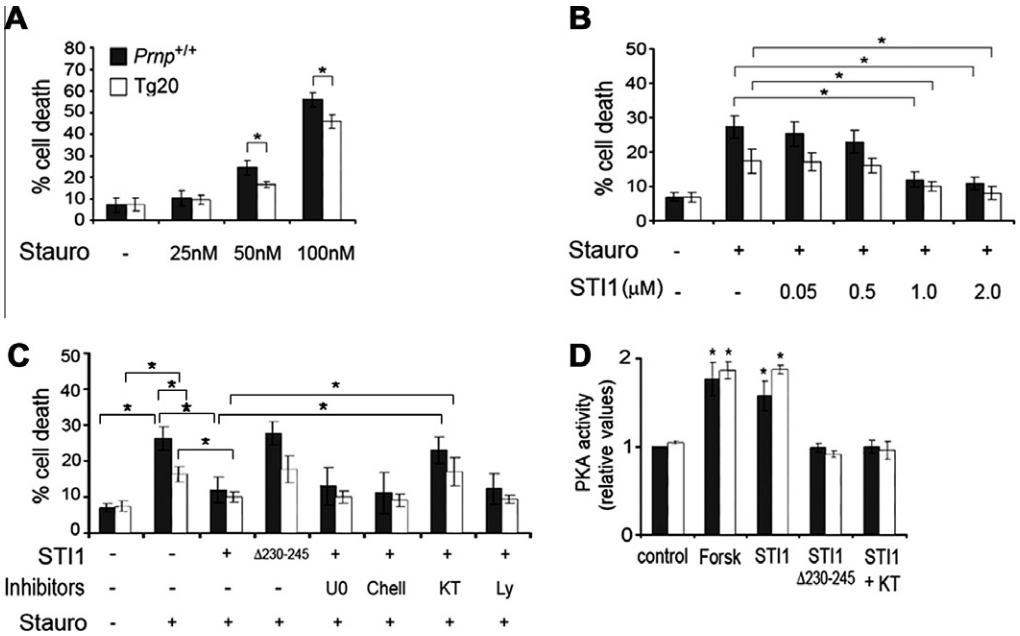

Fig. 2. PrP^C expression modulates astrocyte survival through interaction with STI1 via the PKA pathway. (A) Dose–response curve of cell death induced by staurosporine treatment: primary astrocytes were incubated with staurosporine (stauro) (25, 50, or 100 nM) for 16 h. (B) Astrocytes were pre-incubated with STI1 (0.05, 0.5, 1.0 or 2.0 μM) for 2 h, followed by staurosporine (50 nM) for 16 h. (C) Astrocytes were pre-incubated with STI1 or STI1$\Delta_{230-245}$, followed by staurosporine for 16 h. Cell cultures were treated with signaling enzyme inhibitors 1 h before incubation with STI1. (D) Astrocytes were pre-incubated with IBMX and treated with forskolin (forsk), STI1, STI1$\Delta_{230-245}$ or STI1 plus KT5720 and PKA activity was determined. ■ *Prnp*$^{+/+}$ primary astrocytes; □ *Tg20* primary astrocytes. $^*P < 0.05$.

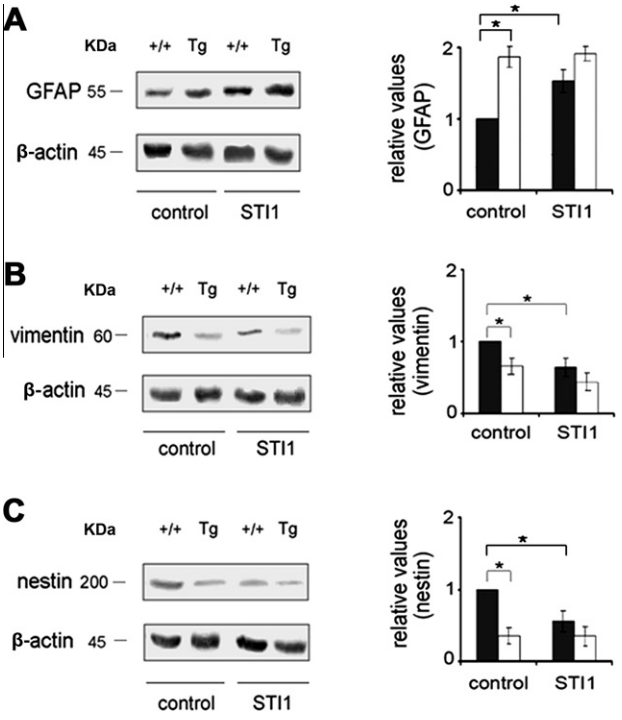

Fig. 3. PrP^C over-expression increases GFAP expression and decreases vimentin and nestin levels in vitro. Primary astrocyte cultures were treated daily with STI1 for 5 days and incubated for an additional 5 days. Cells were lysed and Western blot assays were conducted (left panels) using anti-GFAP (A), anti-vimentin (B), anti-nestin (C) and anti-β-actin antibodies for loading controls (A–C). The bands obtained after membrane X-ray film exposure were analyzed by densitometric scanning (right panels). Values represent the ratios between GFAP, vimentin, nestin or actin for each sample. *Prnp*$^{+/+}$ (■), Tg: *Tg20* (□). $^*P < 0.05$.

and hypoxic-ischemic brain insult [43]. Therefore, PrP^C levels are directly related with neuroprotection.

In pathological contexts such as in prion diseases, GFAP acts as a key player in cytoarchitectural changes from reactive astrocytosis that is accompanied by an increase in GFAP expression [44]. In our study, levels of astrocytic-PrP^C seem to determine GFAP, vimentin and nestin expression patterns, and, consequently, the astrocyte differentiation profile. It is important to notice that although *Tg20* astrocytes present an increase of GFAP expression, they remain protoplasmatic (*flat*) on culture which is a typical morphology of non-reactive astrocytes. This is consistent with previous in vitro studies showing lower GFAP levels and higher vimentin and nestin expression in PrP^C-null astrocytes related to WT cells [5], as well as the fact that PrP^C expression is positively correlated to the differentiation of multipotent neural precursors [45,46].

ERK1/2 activity and GFAP expression levels were increased, whereas vimentin and nestin expression decreased in PrP^C over-expressing astrocytes when compared to WT astrocytes. Similarly to our data, the thyroid hormone induces astrocyte differentiation with MAPK/ERK activation [47] and an increase in GFAP expression and decrease of vimentin levels [35,48]. In this context, the activation of formyl peptide receptor 1 (FPRL1) requires MAPK/ ERK phosphorylation and triggers an increase in GFAP production in human U87 astrocytoma cells [49]. On the whole, these data suggest that high MAPK/ ERK activity levels are related to an increase of GFAP expression and decrease of vimentin and nestin expression in Tg20 cells, resulting in astrocyte maturation.

The present results show that STI1 promotes astrocyte survival through the PKA pathway in both *Prnp*$^{+/+}$ and *Tg20* mice. These results are in accordance to previous data [5] and indicate the involvement of the PKA pathway in the protection against staurosporin-induced astrocyte death promoted by STI1 interaction with PrP^C. Staurosporin treatment is known to increase expression of pro-apoptotic oncogene Bax and reduce Bcl-2 expression [37–40]. However, it is unknown whether these pathways are also affected by STI1.

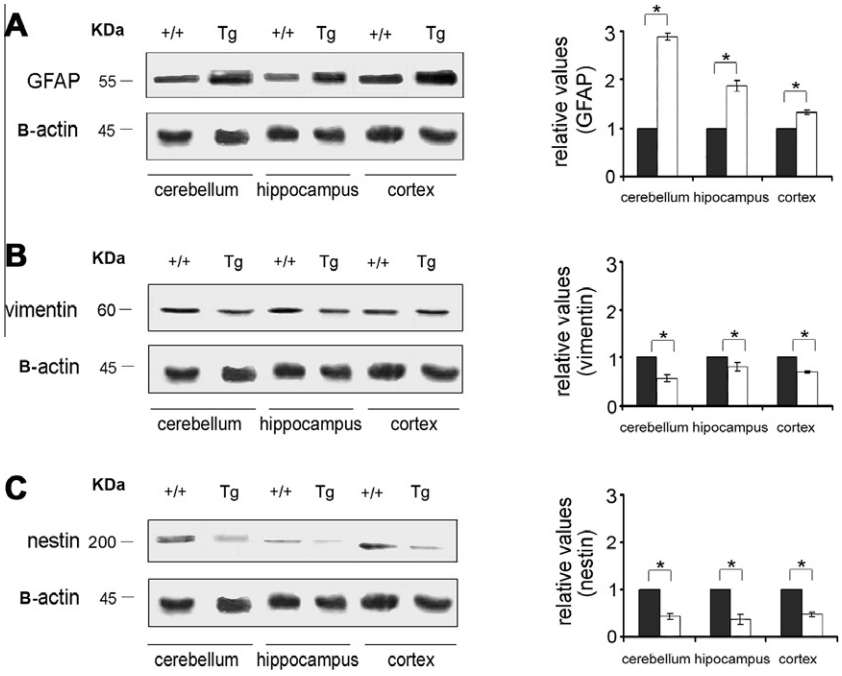

Fig. 4. PrP^C over-expression modulates GFAP, vimentin and nestin expression levels in vivo. E18 brain extracts from cerebellum, cerebral cortex and hippocampus were lysed and the proteins were submitted to Western blot assays using anti-GFAP (A), anti-vimentin (B), anti-nestin (C) and anti-β-actin as loading controls (A–C) antibodies (left panels). The bands obtained after membrane X-ray film exposure were analyzed by densitometric scanning (right panels). Values represent the ratios between GFAP, vimentin, nestin or actin for each sample. *Prnp*$^{+/+}$ (■), Tg: *Tg20* (□) extracts from cerebellum, hippocampus or cerebral cortex. $^*P < 0.05$.

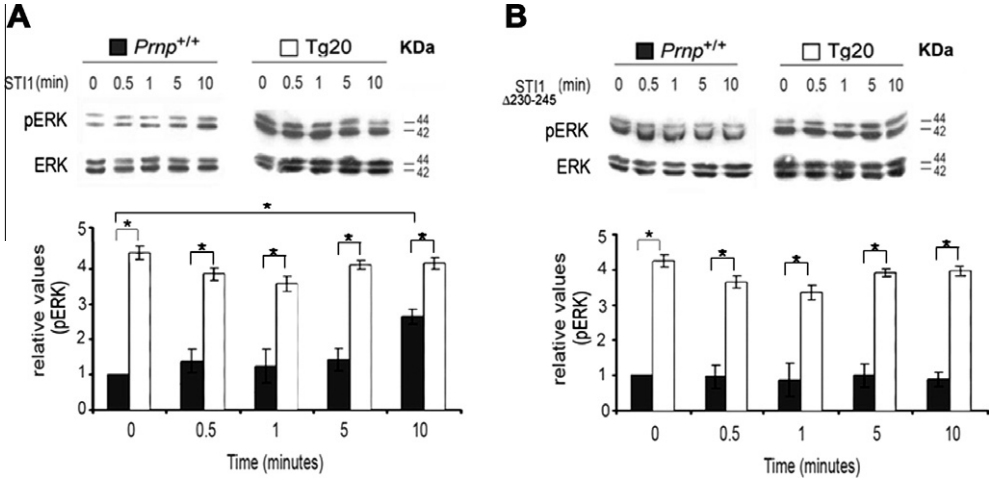

Fig. 5. ERK 1/2 activity is increased in PrP^C over-expressing astrocytes. ERK1/2 phosphorylation assays: astrocyte cultures were stimulated with 1.0 μM STI1 (A) or STI1$\Delta_{230-245}$ (B) for 0, 0.5, 1, 5 and 10 min. Western blots were performed using anti-phospho-ERK1/2 and anti-total ERK1/2 antibodies. Densitometric analyses of bands obtained after membrane X-ray film exposure are shown in the upper panels. Lower panel values represent the ratios between phospho-ERK1/2 (pERK) and total ERK1/2 (ERK) for each sample. *Prnp*$^{+/+}$ (■), *Tg20* (□). $^*P < 0.05$.

We have also verified that the STI1 treatment promotes an increase in GFAP expression and a decrease of vimentin and nestin expression in WT astrocytes but not in PrP^C over-expressing cells. The high PrP^C concentration in Tg20 astrocytes could be responsible for the increased ERK1/2 basal activity and prevents STI1 effects upon the expression of cytoskeleton proteins. Indeed, Tg20 astrocytes already show higher GFAP expression and lower vimentin and nestin expression in relation to WT astrocytes before STI1-treatment, suggesting that *PrP^C* over-expression in astrocytes does accelerate cell maturation independent of STI1-treatment. PrP^C has multiple ligands [7] and the high PrP^C levels in Tg20 astrocytes could facilitate the association of this protein with other partners besides STI1, to promote astrocyte maturation.

In accordance to our studies, it is interesting to note that *PrP^C-null* astrocytes were more sensitive [5] and *Tg20* more resistant to staurosporine-induced cell death than WT cells (present data), which suggests that that PrP^C is a key element in the promotion of astrocyte survival. Furthermore, we have demonstrated that PrP^C is a response mediator for STI1 in astrocyte survival and a relevant factor in the modulation of GFAP/vimentin/nestin expression, which are involved in astrocyte differentiation.

The present study complements previous studies [5] and suggests that high PrP^C levels increase resistance to glial death and promote astrocyte maturation during development. Our group has demonstrated that PrP^C expression in astrocytes during development is significant for sustaining cell-to-cell interactions, the

organization of the extracellular matrix and the secretion of trophic factors, all of which are essential events for neuronal development [11]. In a pathological context, astroglial PrP^C is able to impair the process of neurodegeneration induced by a truncated prion protein [46,50]. On the other hand, PrP^C expression only in astrocytes is sufficient to cause neuronal damage upon prion infection [51]. PrP^C levels in astrocytes are important not only to modulate astrocyte development but also in the neuron–glia interaction and can be associated to prion disease progression. Therefore, studies regarding PrP^C expression associated with its functions in glial cells are relevant and should be further investigated.

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